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Sequence-Selective DNA Binding with Cell-Permeable Oligoguanidinium-Peptide Conjugates

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Conjugation of a short peptide fragment from a bZIP protein to an oligoguanidinium tail results in a DNA-binding miniprotein that selectively interacts with composite sequences containing the peptide-binding site next to an A/T-rich tract. In addition to stabilizing the complex with the target DNA, the oligoguanidinium unit also endows the conjugate with cell internalization properties.

Transcription Factors (TFs) are specialized proteins that participate in the regulation of gene expression by binding to key DNA regulatory sequences,[1] and thereby promoting or inhibiting the assembly of the transcriptional complex.[2] According to this fundamental role, alterations in the activity of TFs are at the origin of many diseases, including cancer.[3] In this context, there has been a great interest in the development of synthetic miniproteins that can reproduce the DNA recognition properties of natural TFs.[4]

It is known that efficient DNA recognition typically involves the cooperative action of multiple protein domains,[5] and isolated monomeric modules of TFs usually fail to interact with their DNA targets. This complicates the development of minimized synthetic versions of natural TFs.[6] One of the most successful strategies to make miniature DNA binding proteins consists of combining the DNA binding domain or fragment of a natural TFs with a small molecule, such as intercalators or minor groove binders.[7] Following this approach, our group has shown that linking the basic regions of bZIP TFs, or single units of zinc finger DNA binders to distamycin or bisbenzamidine derivatives affords conjugates that display high affinity for specific DNA sequences of up to nine base pairs.[8] On the other hand, a major obstacle in translating these synthetic binders to a real cellular context derives from their poor cellular uptake. In recent years, the discovery of cell-penetrating peptides (CPPs) has opened new opportunities for transporting designed cargoes inside living cells.[9] While most CPPs consist of arginine-rich peptidic sequences, non-peptidic bicyclic guanidinium oligomers have also demonstrated good translocation properties.[10] In addition to their potential transport applications, qualitative molecular modeling suggests that the periodicity of hydrogen bond donors in these oligomers might fit that of the phosphate backbone in a double stranded DNA, thus favoring DNA recognition (Figure 1b).[11] Therefore, we envisioned that these guanidinium oligomers could be used as electrostatic anchors for the stabilization of the DNA complexes of TF fragments that otherwise would not bind to their target site. Indeed, many DNA-binding proteins make use of short oligocationic peptide tails to increase the thermodynamic stabilization of their complexes with the DNA.[12] Moreover, we expected that the resulting hybrids might be able to cross cell membranes.

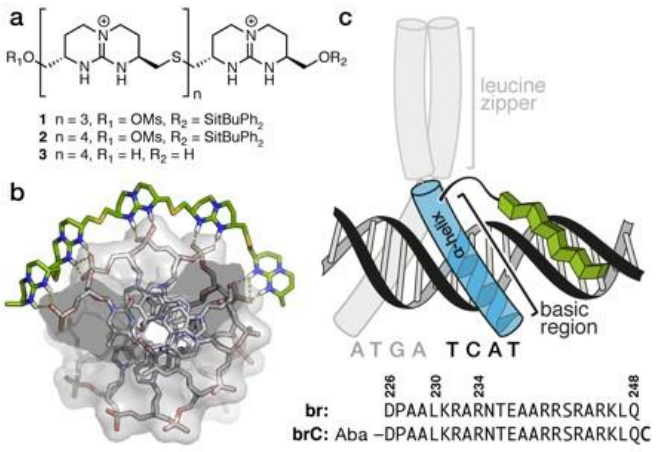


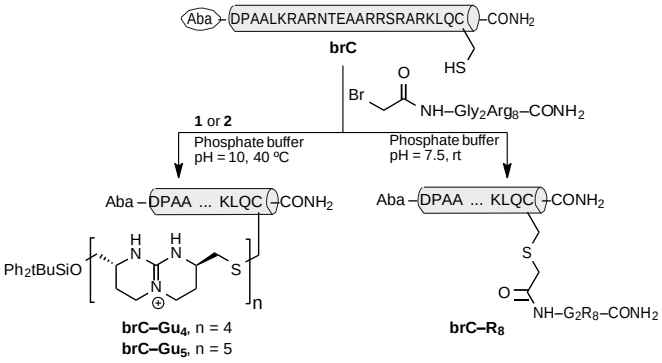
Fig 1. (a) Oligoguanidines used in this study. (b) Structural proposal of the interaction between an oligoguanidine and the DNA; view along the DNA axis showing potential hydrogen bonds. (c) Cartoon representation of a designed hybrid bound to the DNA. The sequences of the GCN4 basic region (**br**) and the target peptide (**brC**) are also shown.

Herein, we report the synthesis, DNA binding and cell internalization studies of conjugates between a protein fragment of a bZIP TF, and bicyclic oligoguanidiums (Figure 1c). These studies revealed that the presence of the guanidinium oligomer not only allows the efficient DNA recognition by the peptidic unit, but also provides for a

sequence selective interaction in the DNA minor groove. In addition, we demonstrate that, in contrast to the parent peptide, the oligoguanidinium conjugate is efficiently internalized by cells.

Our design is based o GCN4, an archetypical bZIP TF that specifically binds to AP1 (5'–ATGA(c)TCAT–3') or ATF/CREB (5'–ATGA(c/g)TCAT–3') sites.[13] The DNA contact takes place through the basic regions, which comprise ~20 N-terminal residues and fold into α-helices only upon binding to their target DNA.[14] Isolated basic regions are unable to interact with their DNA target site with reasonable affinity. We synthesized a GCN4 fragment comprising residues Asp226 to Gln248 (**br**).[15] This core sequence was extended with a cysteine residue at the C-terminus as nucleophilic handle for the attachment of the oligoguanidium fragments.

The peptide **brC** was synthesized following standard Fmoc solid phase peptide synthesis protocols, and purified by HPLC. As electrophilic coupling partners we chose the mesylates **1** and **2**, featuring either tetra- (**Gu$_4$**) or pentaguanidinium (**Gu$_5$**) units.[16] Conjugation between the peptide **brC** and the oligoguanidinium mesylates was performed by heating the corresponding mixtures in phosphate buffer (pH 10) at 40 ºC for 14 h. The desired products, **brC–Gu$_4$** and **brC–Gu$_5$**, were isolated in moderate yields (45 % and 28%, respectively), and identified by ESI-MS. As a control, we also synthesized a conjugate containing an octaarginine instead of the oligoguanidiniums **(brC–R$_8$)**, obtained by incubating at rt the peptide **brC** with an N-terminal bromoacetylated Gly$_2$-Arg$_8$ peptide (Scheme 1 and ESI).



Scheme 1. Key steps in the synthesis of the conjugates of the basic region of GCN4 (**brC**). Aba stands for p-aminobenzoic acid, and is included as a chromophore for spectroscopic monitorization.

Following the synthesis of the oligoguanidinium and arginine conjugates, we studied their DNA binding properties by electrophoretic mobility assays (EMSA) in polyacrylamide gel under non-denaturing conditions, and using ^{32}P-labelled oligonucleotides for radioactive detection. Gratifyingly, incubation of **brC–Gu$_5$** with the double-stranded oligo **AP1^{hs}•A/T**, which contains the AP1 half-site required for peptide binging (TCAT) next to an A/T-rich site (AATTT), gave rise to a new slow-migrating band, consistent with the formation of a well-defined complex (Figure 2, panel A1). Moreover, EMSA titrations demonstrated the high stability of the resulting **brC–Gu$_5$** / **AP1^{hs}•A/T** complex (K_D ≈ 170 nM, see ESI). The **brC–Gu$_4$** analogue is also capable of forming discrete complexes with the composite target DNA, although in this case displaying much weaker binding (K_D ≈ 813 nM, see ESI). It is important to note that the peptide **brC** itself binds the target DNA, under the same conditions, with a very low affinity (~10 μM).[17] Importantly, additional experiments with oligos containing mutations in the A/T-rich site (**AP1^{hs}•mA/T**, sequence: TCAT • GGCCG), or in the peptide binding site (**mAP1^{hs}•A/T**, sequence: CGGC • AATTT), showed that neither **brC–Gu$_4$** nor **brC–Gu$_5$** give rise to retarded EMSA bands (Figure 2, panels B1 and C1, and ESI). Remarkably, the oligoarginine control **brC–R$_8$** induces smeared bands with **AP1^{hs}•A/T**, indicating the formation of a weak 1:1 complex and a number of ill-defined complexes, possibly arising from non-specific electrostatic interactions (Figure 2, panel A2).[18] This effect is even more pronounced in the case of the mutated oligo **AP1^{hs}•mA/T**, which gives rise to heavily smeared bands in the gel (Figure 2, panel B2). As expected, this control conjugate does not show new bands in the presence of the mutated DNA **mAP1^{hs}•A/T** lacking the binding site for the basic region. These data, and particularly the sequence selectivity displayed by **brC–Gu$_4$** and **brC–Gu$_5$**, suggest that the oligoguanidine is not merely working as an electrostatic anchor, but it might also be establishing some sequence-specific interactions, likely through its (partial) insertion into the DNA minor groove. Control experiments with two oligonucleotides containing one or three G/C-base pairs as spacers between the peptide half-site and the A/T-rich region, confirmed the selectivity of the interaction of **brC–Gu$_5$** with the target site (see ESI).

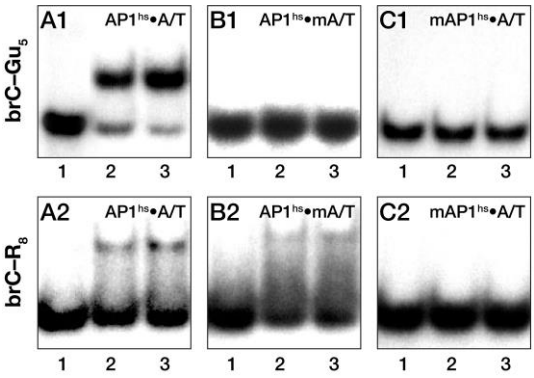


Fig 2. EMSA studies of the binding of **brC–Gu$_5$** and **brC–R$_8$** to different dsDNA (Lanes 1-3 in all panels correspond to 0, 200 and 300 nM of the **brC** conjugate and ≈ 50 nM of each DNA with a small fraction (≈ 0.1%) of ^{32}P-labeled oligo for radioactive detection. Samples were incubated for 30 min in 18 mM Tris (pH 7.0), 50 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM EDTA, 9% glycerol, 0.11 mg/mL BSA and 4.2% NP–40 at 20 ºC. **AP1^{hs}•A/T**: 5'–ACGAACG *TCAT* • AATTT CCTC–3'; **mAP1^{hs}•A/T**: 5'–ACGAACG CGGC • AATTT CCTC–3', **AP1^{hs}•mA/T**: 5'–ACGAACG *TCAT* • GGCCG CCTC–3' (peptide binding site in italics, and minor groove binding site underlined; only one strand shown).

Circular dichroism is particularly suited to detect the interaction of bZIP peptides with DNA, because their binding is coupled to the folding of the basic region into an α-helix [19] Thus, incubation of a 5 μM solution of **brC–Gu$_5$** with one equivalent of the oligonucleotide **AP1^{hs}•A/T** resulted in a large increase of the negative band at 222 nm, which correlates with the expected α-helix folding (Figure 3, left), and is in agreement with the high affinity displayed by this conjugate in the EMSA experiments. There is also an increase in the intensity of the negative band at 222 nm in the presence of the **AP1^{hs}•mA/T** dsDNA , albeit lower than with **AP1^{hs}•A/T**, while this increase is not observed with the DNA lacking the cognate peptide-binding site (**mAP1^{hs}•A/T**) (see the ESI). Importantly, in addition to the folding of the peptide chain, CD experiments revealed a noteworthy distortion of the DNA upon formation of the complex with the **brC–Gu$_5$** conjugate, as evidenced by the changes in the characteristic bands of the B–DNA at 245 and 275 nm (Figure 3, left and

ESI). In contrast, the **brC–R$_8$** conjugate did not induce any change in the CD spectral region of the DNA, but there is folding of the peptide chain (Figure 3, right, and ESI). Interestingly, control CD experiments with the pentaguanidinium **3** also show a decrease in the intensity of the band at 275 nm (figure 3, left). All of these results suggest that the interaction of the guanidinium pentamer in **brC–Gu$_5$** with the DNA is intimate, and induces perturbations in the winding of the DNA helix, perhaps because of a shielding in the electrostatic repulsions between adjacent phosphates by the oligocationic tail.[20]

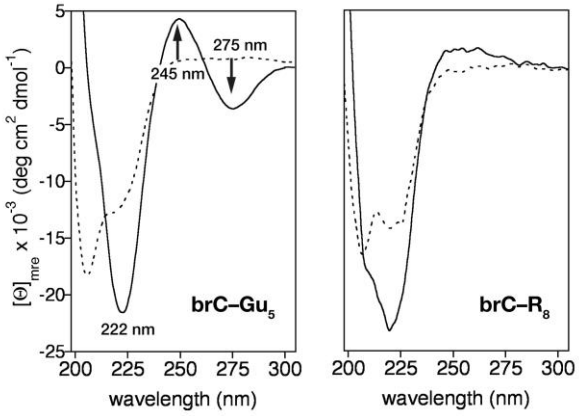


Fig 3. Circular dichroism of 5 μM solutions of **brC–Gu$_5$** and **brC–R$_8$** in absence of DNA (dashed lines), and in the presence of 1 equiv of **AP1^{hs}•A/T** (solid lines). The contribution of the parent DNA to the CD spectrum of the complexes has been subtracted. Samples contained 5 μM of corresponding dsDNA (when present) and 5 μM of peptides in 10 mM phosphate buffer (pH 7.5) and 100 mM of NaCl at 20 ºC.

Taken together, these results indicate that the oligoguanidine hybrids, particularly **brC–Gu$_5$**, interacts efficiently with specific DNA sequences containing the AP1 half site (TCAT) adjacent to an A/T-rich region. The interaction is stronger and more selective than with the hybrid **brC–R$_8$**, despite the fact that this conjugate contains more guanidinium groups. The high affinity and selectivity displayed by **brC–Gu$_5$** suggests that the rigid structure of the bicyclic guanidinium scaffolds might be especially appropriate for complementing the DNA surface. While the preliminary molecular modeling suggested an interaction with the DNA phosphodiester backbone (Figure 1b), this would imply similar binding to different DNAs as long as they have the peptide-binding site. However, the experimental results indicate that **AP1^{hs}•A/T** gives rise to more stable complexes than **AP1^{hs}•mA/T**. Since it is known that many DNA binders interact with A/T-rich sites by inserting guanidinium or amidinium groups in the DNA minor groove,[21] we hypothesized that the binding preference of our conjugates for DNAs featuring A/T-rich tracts might result from the insertion of some of the guanidinium moieties into the minor grove of these sequences.

Competition assays show that the water-soluble pentaguanidine **3** is capable of displacing a fluorescent bisbenzamidine probe from the A/T-rich minor groove of ds-oligos (see the ESI). [22] Not surprisingly, this displacement is highly dependent on the ionic strength of the medium, so that a low salt buffer containing 30 mM of NaCl induced the formation of more stable complexes (K_D ≈ 9 nM), whereas increasing the concentration of NaCl to 100 mM results in a marked decrease in the affinity (K_D ≈ 140 nM). These results suggest that the pentaguanidine **3** binds to dsDNAs containing A/T-rich tracts through partial insertion of a number of bicyclic units in the minor groove and simultaneous formation of salt bridges between the terminal units and the phosphodiester backbone. This mechanism would account for the sequence selectivity observed with the conjugate, as well as for the high influence of the ionic force.

We finally carried out preliminary cell internalization tests using Vero cells and tetramethylrhodamine (**TMR**) derivatives of the GCN4 basic region, **TMR–brC**, and its conjugates **TMR–brC–R$_8$** and **TMR–brC–Gu$_5$** (see the ESI). Thus, while **TMR–brC** is essentially not internalized (Figure 4A), the two conjugates led to a clear increase in the intracellular fluorescence (Figures 4B and 4C). The conjugates, which are not significantly cytotoxic (see the ESI), appear to be trapped in endocytic vesicles (Figures 4D-F). The future use of these compounds as genetic tools might require the incorporation of groups that could favor their endosomal escape and subsequent translocation into the nucleus. [23]

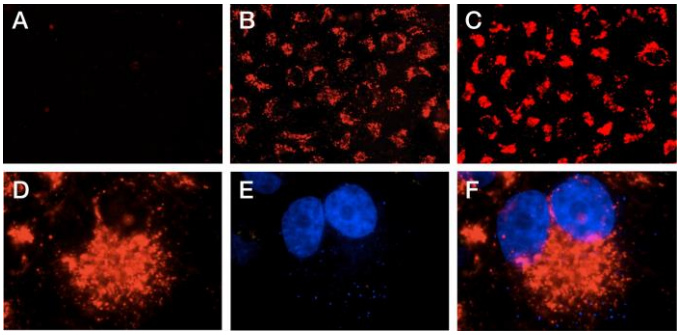


Fig 4. Fluorescence microscopy images of Vero cells incubated for 45 min at 37 ºC. Top: Micrographs taken at 400X, ISO 400. (A) 5 μM of **TMR–brC**. (B) 5 μM of **TMR–brC–R$_8$**. (C) 5 μM of **TMR–brC–Gu$_5$**. Bottom: 5 μM of **TMR–brC–Gu$_5$** and co-staining with 2.5 μM of DAPI. at 1000X, ISO 400. (D) Red channel. (E) Blue channel. (F) Overlay of D and E.

Conclusions

In conclusion, tethering oligoguanidinium fragments to monomeric basic regions of a bZIP protein allows recovery of its interaction to a DNA consensus site, provided it is adjacent to A/T rich tracts. This A/T selectivity stems from the intrinsic preference of the oligoguanidinium moiety to bind these sequences, most probably through insertion of some of its bicyclic units in the minor groove, while the others act as electrostatic phosphate clamps. The oligoguanidinium appendage, in addition to promote DNA binding, allows an efficient cellular uptake.

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